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(54) **STABILIZED MUTANT OPSIN PROTEINS**

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(57) **ABSTRACT**

Methods and compositions for stabilizing opsin protein, such
as a Pro23His mutant opsin protein, in a vertebrate visual
system, by administration of opsin-binding synthetic retin-
oids, are provided. The mutant opsin protein binds to the
synthetic retinoid, which stabilizes the mutant opsin protein
and/or ameliorates the effects of the mutant opsin protein on
the vertebrate visual system.

20 Claims, 7 Drawing Sheets

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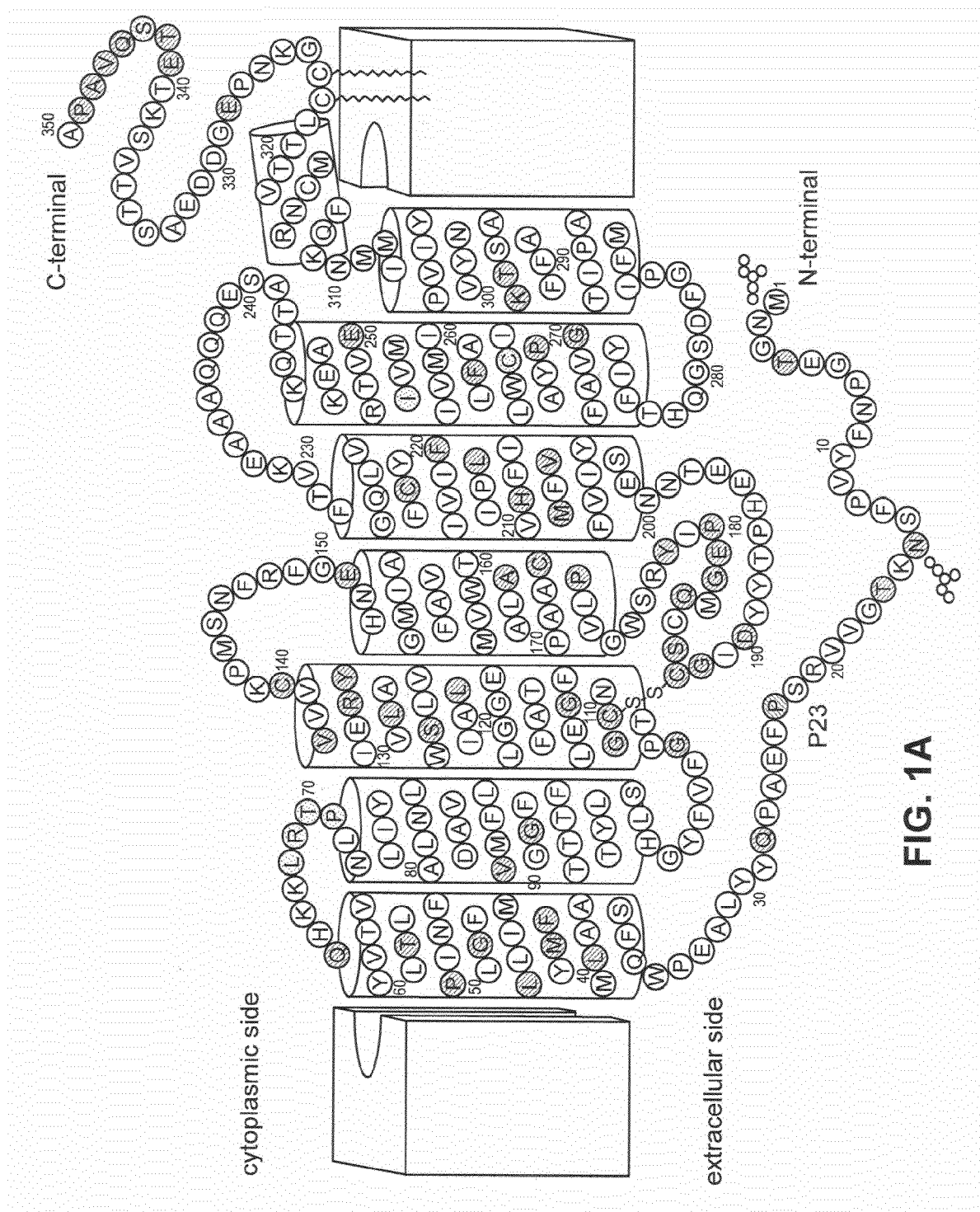
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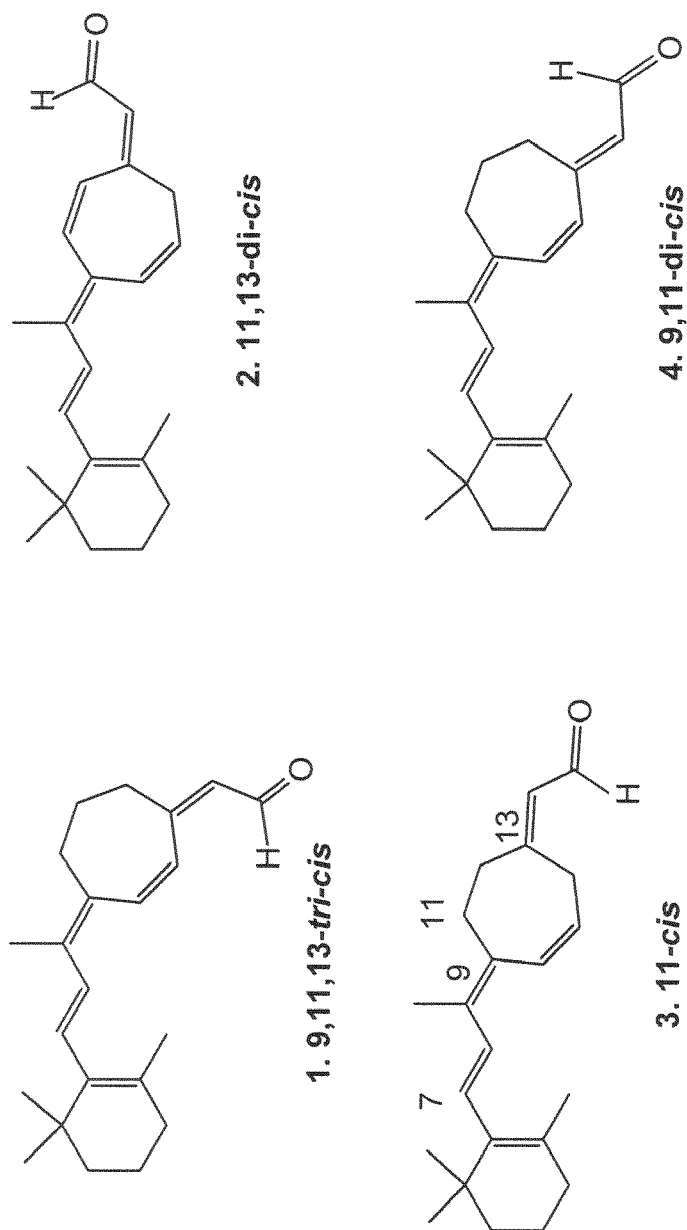
FIG. 1A
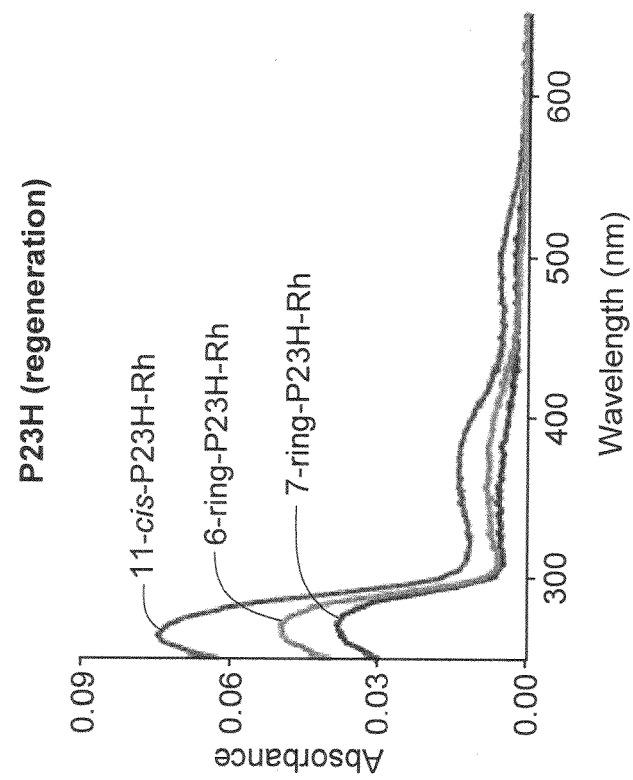
2. 11,13-di-cis

4. 9,11-di-cis

1. 9,11,13-tri-cis

3. 11-cis

FIG. 1B

P23H (regeneration)

11-*cis*-P23H-Rh
6-ring-P23H-Rh
7-ring-P23H-Rh

Wavelength (nm)

Absorbance

FIG. 2B

WT (regeneration)

11-*cis*-Rh (498 nm)
7-ring-Rh (494 nm)
6-ring-Rh (502 nm)

Wavelength (nm)

Absorbance

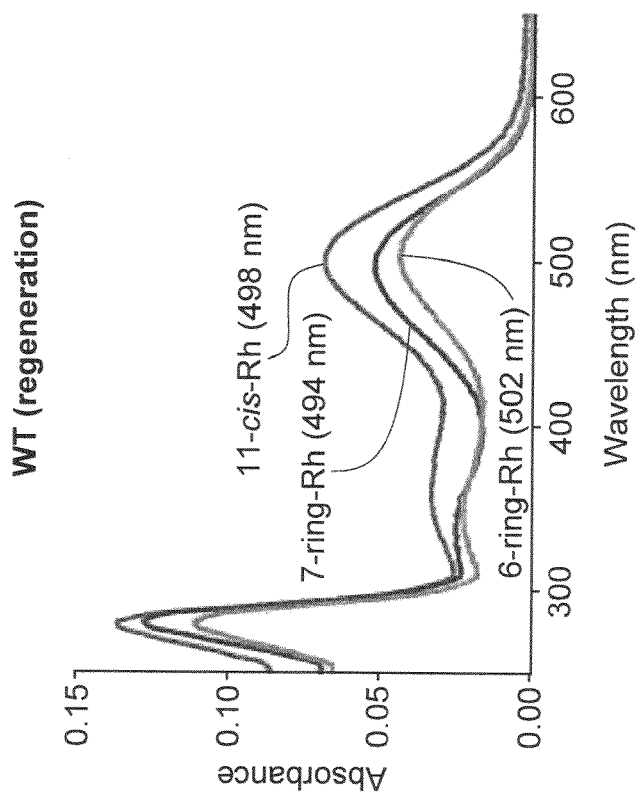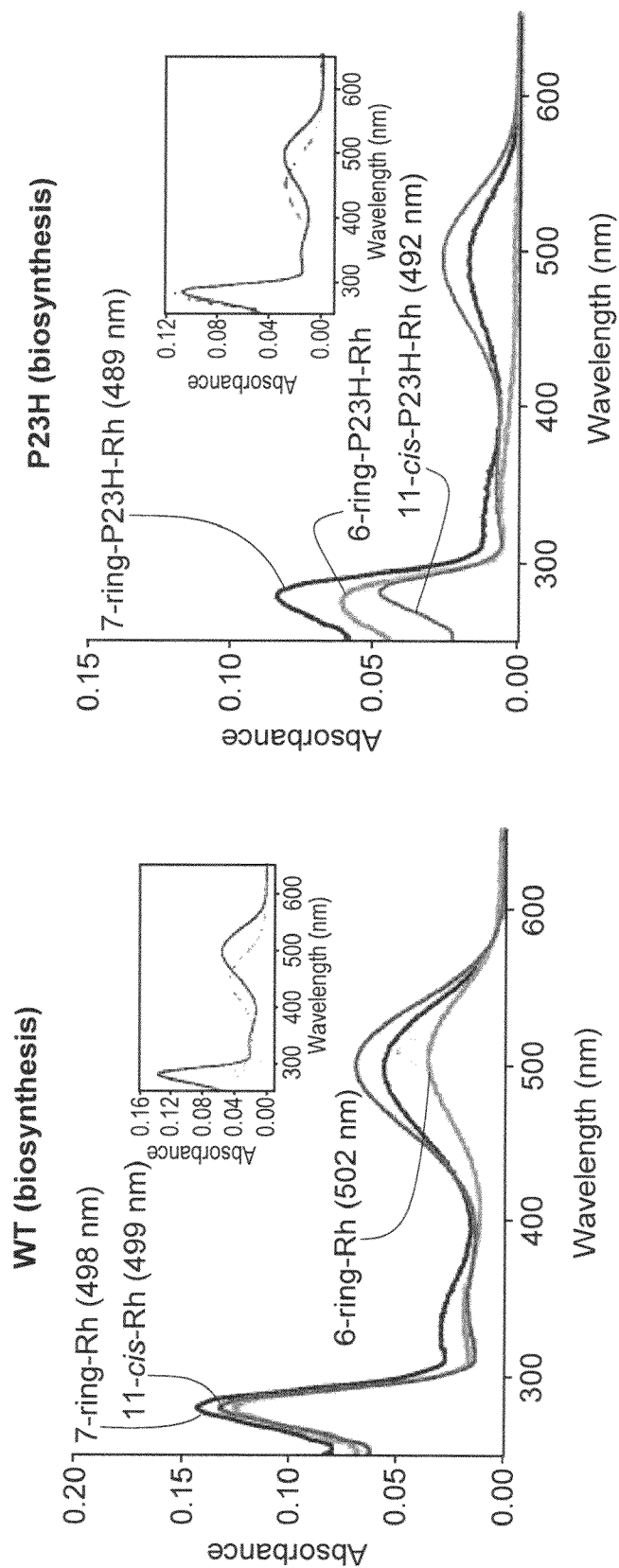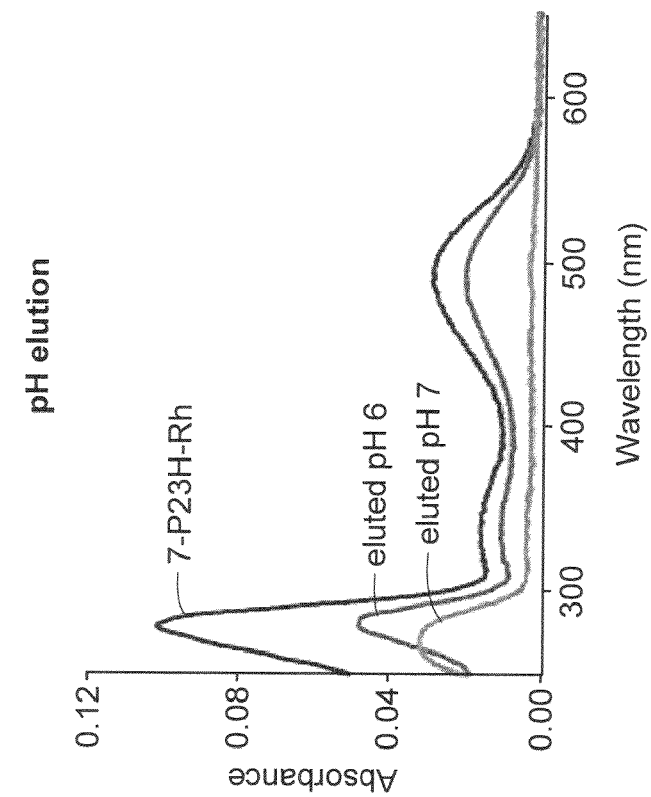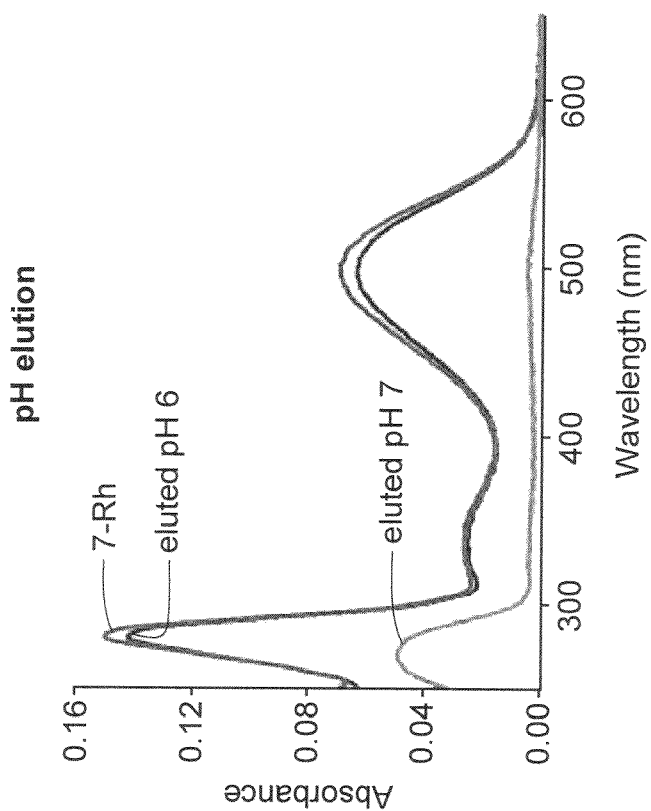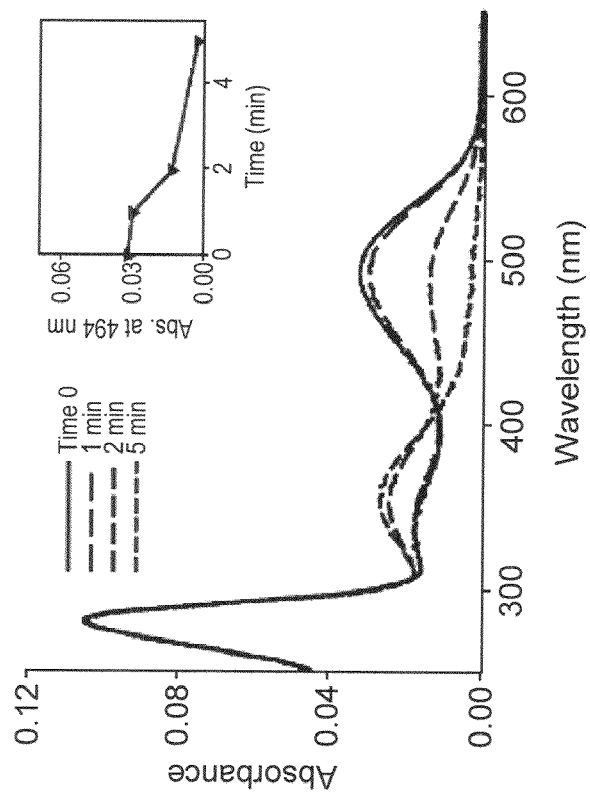**FIG. 2A**

FIG. 2D

FIG. 2C

FIG. 2F

FIG. 2E

FIG. 3B

FIG. 3A
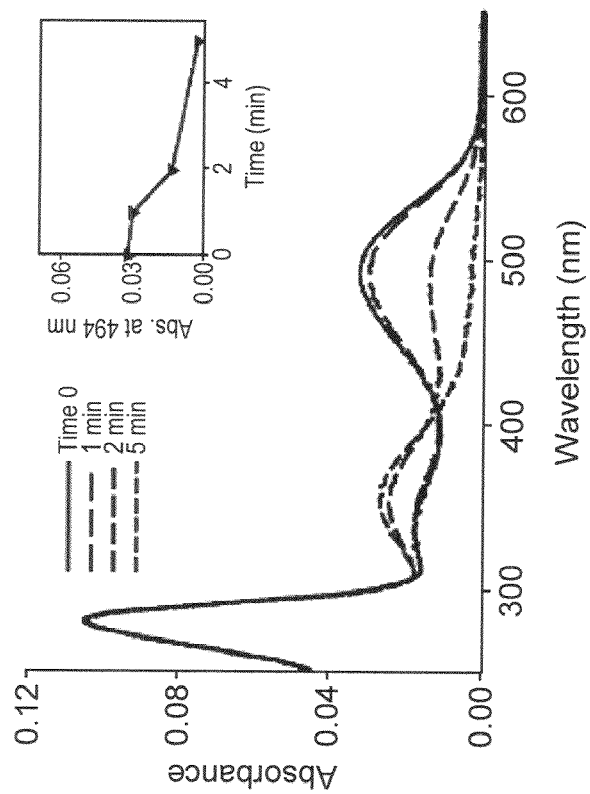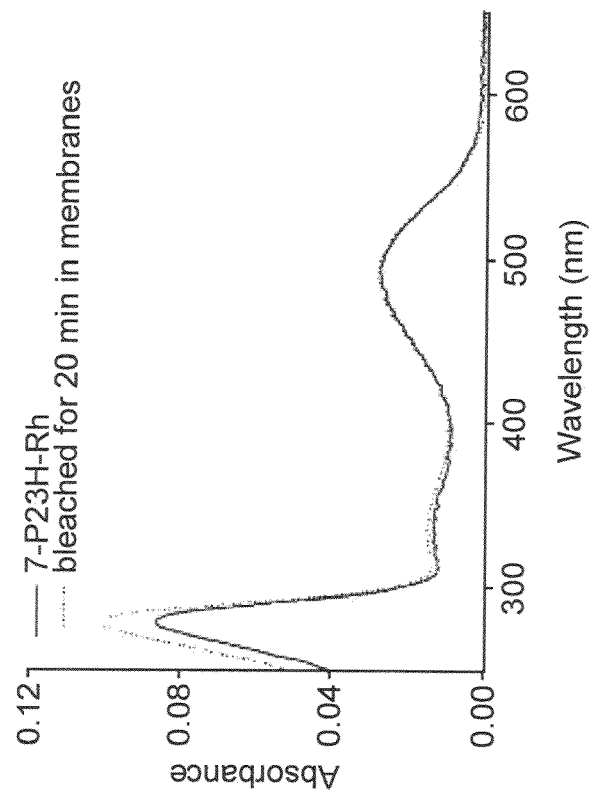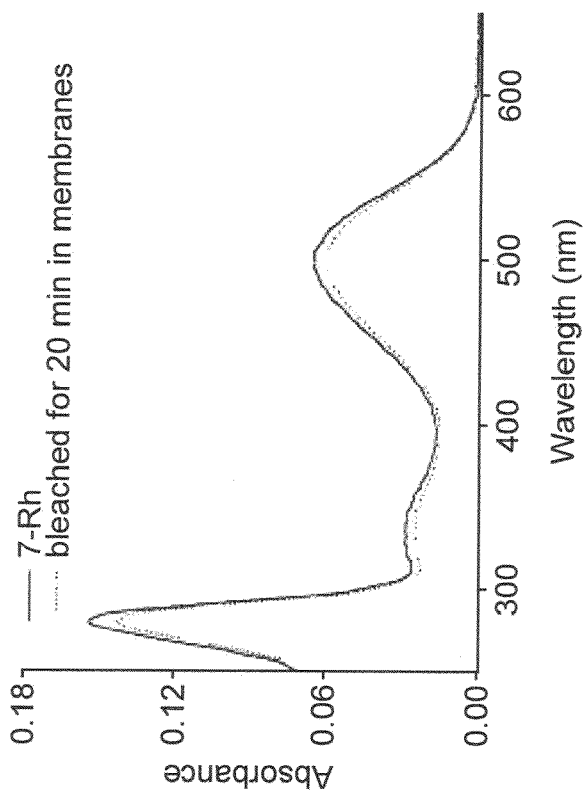
FIG. 3D

FIG. 3C

STABILIZED MUTANT OPSIN PROTEINS

CROSS-REFERENCES TO RELATED APPLICATIONS

This application is a continuation of U.S. application Ser. No. 10/801,078 filed Mar. 15, 2004, pending, which claims the benefit of U.S. Provisional Patent Application No. 60/455, 182, filed Mar. 14, 2003, the disclosures of which are incorporated by reference herein.

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made with government support under grant numbers EY008061 and EY009339 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

A diminished visual acuity or total loss of vision may result from a number of eye diseases or disorders caused by dysfunction of tissues or structures in the anterior region of the, eye and/or posterior region of the eye. The eye is divided anatomically into an anterior and posterior segment. The anterior segment includes the cornea, anterior chamber, iris and ciliary body (anterior choroid), posterior chamber and crystalline lens. The posterior segment includes the retina with optic nerve, choroid (posterior choroid) and vitreous. The posterior portion of the eyeball supports the retina, choroid and associated tissues.

Protein conformational disorders are a set of inherited human diseases in which mutant proteins are misfolded. Protein conformation disorders can affect proteins of the vertebrate visual system and cause dysfunction of tissues or structures within the eye. For example, misfolded proteins can aggregate, causing damage within cells expressing the mutant protein. Certain types of retinitis pigmentosa are associated with protein conformational disorders. For example, many opsin mutants associated with retinitis pigmentosa are misfolded and retained within the cell.

Rhodopsin (Rh) is the visual pigment protein of the rod cell and belongs to a large family of transmembrane G protein-coupled receptors, which are involved in numerous physiological functions. This superfamily of membrane glycoprotein receptors is characterized by seven transmembrane α-helices that are anchored within the lipid bilayer. Rhodopsin is composed of opsin, the apoprotein, which is bound to chromophore. In contrast to other G protein-coupled receptors, which respond to diffusible ligands, rhodopsin responds to light. The chromophore in rhodopsin, 11-cis-retinal, is a covalently bound reverse agonist that yields a distinct UV-visible spectrum with a max of approximately 500 nm. Rhodopsin is approximately 40.000 daltons. The crystal structure of rhodopsin has been previously elucidated.

In recent years, more than 100 opsin mutants have been linked to various genetic forms of retinitis pigmentosa, the most common form of hereditary retinal degeneration. Retinitis pigmentosa leads to photoreceptor death and subsequent severe loss of peripheral and night vision. These opsin mutants account for nearly 50% of all the autosomal dominant retinitis pigmentosa cases with the most common being a substitution of Proline 23 to Histidine (P23H), which accounts for approximately 10% of all retinitis pigmentosa cases.

Although opsin mutants display distinct biochemical features, the mutant phenotypes fall mainly into three basic classes. Class I mutants are expressed at nearly wild-type levels and form stable pigment with 11-cis-retinal in the dark. The associated amino acid substitutions duster at the C terminus of rhodopsin and disrupt vectorial transport to the rod outer segment. Some mutants also inefficiently activate transducin. Class II mutants do not bind 11-cis-retinal and are retained in the endoplasmic reticulum. Class III mutants, like P23H, form small amounts of pigment and mainly remain in the endoplasmic reticulum or form aggresomes. These mutants are typically targeted for degradation by the ubiquitin proteasome system. Other phenotypic properties associated with opsin amino acid substitutions may include the destabilization of the structure formed within the rod outer segment by mutated rhodopsin molecules or the disruption of other biological processes unique to the rod outer segment.

Patients with the P23H substitution usually have milder disease progression than those harboring other rhodopsin mutations. Based on the crystal structure of rhodopsin, Proline 23 is located in the N-terminal tail within one of the β-strands that make up an integral part of the N-terminal plug. The plug keeps the chromophore in its proper position, and mutations within this region result in improper folding of opsin and poor binding of the chromophore. P23H-opsin forms the pigment poorly, does not acquire the Golgi-related glycosylation, and is retained within the cell, collectively providing evidence that it is misfolded.

There is currently no effective treatment for protein conformational disorders of vertebrate visual systems. The present invention satisfies this and other needs.

BRIEF SUMMARY OF THE INVENTION

The present invention provides methods of restoring or stabilizing photoreceptor function in a vertebrate visual system comprising a mutant Opsin protein. Mutant opsin proteins can be stabilized by contacting the mutant protein with an opsin-binding synthetic retinoid. The mutant opsin protein binds to the synthetic retinoid, which stabilizes the mutant opsin protein and/or ameliorates the effects of the mutant opsin protein on the vertebrate visual system.

In one aspect, a method is provided for restoring photoreceptor function in a vertebrate eye having a mutant opsin protein. The method generally includes administering to the vertebrate an effective amount of an opsin-binding synthetic retinoid in a pharmaceutically acceptable vehicle. The opsin-binding synthetic retinoid binds to and stabilizes the opsin protein in the eye. The opsin-binding synthetic retinoid can be, for example, an 11-cis-7-ring retinal, a 9-cis-7-ring retinal, cycloheptatrienylidene 11-cis-locked retinal or cycloheptatrienylidene 9-cis-locked retinal. The opsin-binding synthetic retinoid also can be, for example, a synthetic retinoid of formula I, II, III, IV, V, VI, VII, VIII, IX, X, XI, XII or XIII. In another embodiment, the opsin-binding synthetic retinoid is a 9-cis-fused retinal. The mutant opsin protein can be, for example, a Pro23His mutant opsin protein.

The opsin-binding synthetic retinoid can be locally administered to the eye. For example, the opsin-binding synthetic retinoid can be locally administered by eye drops. intraocular injection or periocular injection. The opsin-binding synthetic retinoid also can be orally administered to a subject comprising the vertebrate eye.

In another aspect, a method is provided for stabilizing mutant opsin protein. The method generally includes contacting the mutant opsin protein with an opsin-binding synthetic retinoid for an amount of time sufficient for the formation of

a stabilized opsin/synthetic retinoid complex. The opsin-binding synthetic retinoid can he. for example, a synthetic retinoid of formula I, II, III, IV, V, VI, VII, VIII, IX, X, XI, XII or XIII. In certain embodiments, the opsin-binding synthetic retinoid is a 9-cis-locked retinal, an 11-cis-locked retinal. an 11-cis-7-ring retinal or a 9-cis-ring retinal. In certain other embodiments. the opsin-binding synthetic retinoid is cyclo-heptatrienylidene 11-cis-locked retinal or cycloheptatrie-nylidene 9-cis-locked retinal. The mutant opsin protein can be, for example, a Pro23His mutant opsin protein.

In yet another aspect, a method of ameliorating loss of photoreceptor function in a vertebrate eye is provided. The method generally includes prophylactically administering an effective amount of an opsin-binding synthetic retinoid in a pharmaceutically acceptable vehicle to a vertebrate eye comprising a mutant opsin protein having a reduced affinity for 11-cis-retinal. The synthetic retinoid binds to and stabilizes the mutant opsin protein.

The opsin-binding synthetic. retinoid can be orally administered to a vertebrate. The opsin-binding synthetic retinoid also can be locally administered to the vertebrate eye.

The opsin-binding synthetic retinoid can be, for example, a synthetic retinoid of formula I, II, III, IV, V, VI, VII, VIII, IX, X, XI, XII or XIII. In certain embodiments, the opsin-binding synthetic retinoid can be a 9-cis-7-ring retinal, an 11-cis-7-ring retinal, cycloheptatrienylidene 11-cis-locked retinal or cycloheptatrienylidene 9-cis-locked retinal. The mutant opsin protein can. for example, have mutation (e.g., an amino acid substitution, deletion, insertion, or the like) in the N-terminal plug. For example, the mutant opsin protein can be a Pro23H is mutant opsin protein.

In yet another aspect, an ophthalmologic composition including an opsin-binding synthetic retinoid in a pharmaceutically acceptable vehicle is provided. The opsin-binding synthetic retinoid can be, for example, a synthetic retinoid of formula I, II, III, IV, V, VI, VII, VIII, IX, X, XI, XII or XIII. The opsin-binding synthetic retinoid can be, for example, a 9-cis-7-ring retinal, an 11-cis-7-ring retinal, cycloheptatrie-nylidene 11-cis-locked retinal or cycloheptatrienylidene 9-cis-locked retinal.

In a related aspect, an oral dosage form including an opsin-binding synthetic retinoid in a pharmaceutically acceptable vehicle is provided. The opsin-binding synthetic retinoid can be, for example, a synthetic retinoid of formula I, II, III, IV, V, VI, VII, VIII, IX, X, XI, XII or XIII. The opsin-binding synthetic retinoid can be, for example, a 9-cis-7-ring retinal, an 11-cis-7-ring retinal, cycloheptatrienylidene 11-cis-locked retinal or cycloheptatrienylidene 9-cis-locked retinal.

In yet another aspect, a method of identifying an opsin-binding synthetic retinoid to stabilize a mutant opsin protein is provided. The method generally includes providing an expression system for the expression of a mutant opsin protein, contacting the mutant opsin protein with a synthetic retinoid for a time sufficient and in suitable conditions for the binding of the synthetic retinoid by the mutant opsin protein; and detecting whether the mutant opsin protein binds the synthetic retinoid to form a stable mutant opsin protein/synthetic retinoid complex. The expression system can he. for example, a eukaryotic cell line expressing the mutant opsin protein. The synthetic retinoid can be, for example. administered to cell culture media in which the eukaryotic cell line is cultured. The opsin-binding synthetic retinoid can be. for example. a synthetic retinoid of formula I, II, III, IV, V, VI, VII, VIII, IX, X, XI, XII or XIII.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. Models of rhodopsin and structures of 7-locked-retinals. FIG. 1A, two-dimensional model of rhodopsin.

White letters on a black background indicate a mutation associated with retinitis pigmentosa. Proline 23 is shown by a black background and the designation "P23." FIG. 1B, chemical structures of the four 11-cis-7-ring retinal isomers. Carbon atom numbering is as accepted in retinoid nomenclature.

FIG. 2. Pigment formation when the chromophores were added to harvested cell membranes or provided during biosynthesis. FIG. 2A, regeneration of wild-type rhodopsin in cell membranes with 11-cis-retinal, 11-cis-6-ring retinal, and 11-cis-7-ring retinal. FIG. 2B. lack of regeneration of P23H-rhodopsin in cell membranes with 11-cis-retinal, 11-cis-6-ring retinal, and 11-cis-7-ring retinal. FIG. 2C, regeneration of wild-type rhodopsin with 11-cis-6-ring retinal, 11-cis-7-ring retinal, and 11-cis-retinal when retinals were added during biosynthesis. FIG. 2D, regeneration of P23H-rhodopsin with 11-cis-7-ring retinal and 11-cis-retinal, but not with 11-cis-6-ring retinal, when retinals were added during biosynthesis. Insets in FIGS. 2C and 2D, acid denaturation of purified 7-rhodopsin and 11-cis-7-ring-P23H-rhodopsin, respectively. FIG. 2E, elution of 7-rhodopsin at pH 6.0 and pH 7.0. FIG. 2F, elution of 7-P23H-rhodopsin at pH 6.0 and 7.0.

FIG. 3. Photosensitivity of 7-rhodopsin and 7-P23H-rhodopsin in detergent and in cell membrane. FIG. 3A, purified 7-rhodopsin is stable to light in detergent. FIG. 3B, purified 7-P23H-rhodopsin was bleached after 5 minutes of exposure to light in detergent.

FIGS. 3C and D. 7-rhodopsin and 7-P23H-rhodopsin in cell membranes were not bleached by light. Insets, changes in absorption at 494 nm as a function of time.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides methods of restoring or stabilizing photoreceptor function in a vertebrate visual system having a mutant opsin protein. Mutant opsin proteins can be stabilized by contacting the mutant protein with an opsin-binding synthetic retinoid. The mutant opsin protein binds to the synthetic retinoid, which stabilizes the mutant opsin protein and/or ameliorates the effects of the mutant opsin protein on the vertebrate visual system. An opsin-binding synthetic retinoid can he administered prophylactically or therapeutically to a vertebrate to prevent or reduce damage to the eye associated with the mutant opsin protein. Suitable vertebrates include, for example, human and non-human vertebrates. Suitable non-human vertebrates include, for example, mammals, such as dogs, cats, horses and other domesticated animals.

The mutant opsin protein has one or more amino acid substitutions, deletions, insertions and/or other mutations that affect nascent protein folding, stability, degradation, localization, or the like. Mutant opsin proteins can be, for example, a class I, class II or class III opsin mutant. Typically, the mutant opsin protein exhibits reduced affinity for 11-cis-retinal and/or does not form a stable complex with 11-cis-retinal. In certain embodiments, the opsin mutant is a class III mutant. The opsin mutant also can be, for example, associated with a dominant retinitis pigmentosa condition. In additional embodiments, the opsin mutant comprises an amino acid substitution, insertion and/or deletion in the N-terminal plug of opsin, which results in reduced affinity for 11-cis-retinal. In an exemplary embodiment, the opsin mutant is a Pro23His mutant in human opsin. In additional embodiments, the opsin mutant is a Phe45Leu mutant, a Gly51Val mutant, a Gly51Arg mutant, a Leu125Arg mutant. a Gly114Val mutant, a Gly114Asp mutant, an Ala164Glu mutant, or the like, in human opsin.

The opsin-binding synthetic retinoids are retinals derived from 11-cis-retinal or 9-cis-retinal, or are 9-cis-retinal. In certain embodiments, the "synthetic retinoid" is a "synthetic cis retinoid." Synthetic retinoids according to the present invention can bind to an opsin mutant, and function as an chaperone, or opsin agonist. As used herein, the term "chaperone" refers to a molecule or compound that helps a nascent polypeptide chain fold into the proper conformation, stabilizes the folding protein, protects the nascent polypeptide from making premature or nonproductive contacts, and/or protects the protein from improper modification.

In certain additional embodiments, the synthetic retinoid restores function (e.g., photoreception) to the mutant opsin when it is part of an opsin/synthetic retinoid complex, whereby the mutant opsin/synthetic retinoid complex can respond to photons.

Synthetic retinoids include 11-cis-retinal derivatives or 9-cis-retinal derivatives such as for example, the following: acyclic retinals; retina's with modified polyene chain length, such as trienoic or tetraenoie retinals: retina's with substituted polyene chains, such as alkyl, halogen or heteratom-substituted polyene chains: retinals with modified polyene chains, such as trans- or cis-locked polyene chains, or with, for example, alkene, alkane, alkene or alkyne modifications; and retinals with ring modifications, such as heterocyclic, heteroaromatic or substituted cycloalkane or cycloalkene rings.

In certain embodiments, the synthetic, retinoid can be a retinal of the retinal of the following formula I:

(I)
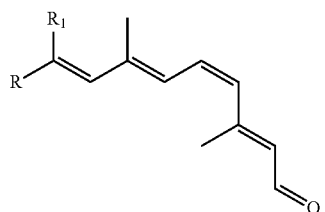
R and R1 can be independently selected from linear, iso-, sec-, tert- and other branched alkyl groups as well as substituted alkyl groups, substituted branched alkyl, hydroxyl, hydroalkyl, amine, amide, or the like. R and R1 can independently be lower alkyl, which means straight or branched alkyl with 1-6 carbon atom(s) such as methyl, ethyl, propyl, isopropyl, butyl, isobutyl, tert-butyl, pentyl, hexyl, or the like. Suitable substituted alkyls and substituted branch alkyls include, for example, alkyls, branched alkyls and cyclo-alkyls substituted with oxygen, hydroxyl, nitrogen, amide, amine, halogen, heteroatom or other groups. Suitable heteroatoms include, for example, sulfur, silicon, and fluoro- or bromo-substitutions.

In certain additional embodiments, R or R1 can be a cyclo-alkyl such as, for example, hexane, cyclohexene. benzene as well as substituted cyclo-alkyl. Suitable substituted cyclo alkyl include, for example, cyclo-alkyls substituted with oxygen, hydroxyl, nitrogen, amide, amine, halogen, heteroatom or other groups. Suitable heteroatoms include, for example, sulfur, silicon, and fluoro- or bromo-substitutions.

The synthetic retinoid also can be a derivative of an 11-cis-retinal or 9-cis-retinal that has a modified polyene chain length of the following formula II:

(II)
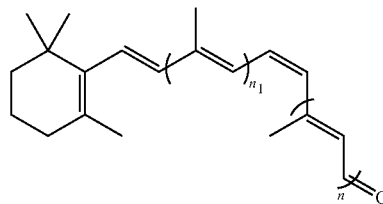
The polyene chain length can be extended by 1, 2, or 3 alkyl, alkene or alkylenc groups. According, to formula II, each n and n_1 can be independently selected from 1, 2, or 3 alkyl, alkene or alkylene groups, with the proviso that the sum of the n and n_1 is at least 1.

The synthetic retinoid also can he a derivative of an 11-cis-retinal or 9-cis-retinal that has a substituted polyene chain of the following formulas, respectively, IIIa and IIIb:
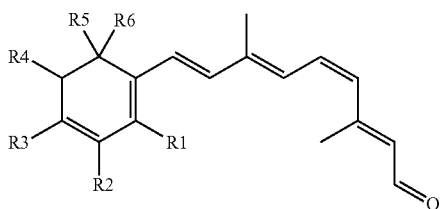
(IIIa)

(IIIb)
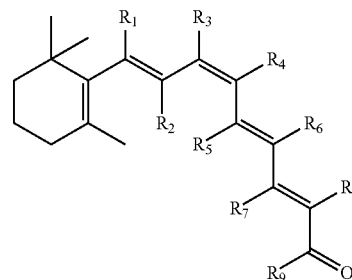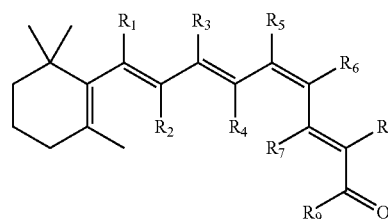
Each of R1 to R9 can be independently selected from hydrogen, alkyl, branched alkyl, cyclo-alkyl, halogen, a heteroatom, hydroxyl, hydroxyalkyl, amine, amide, or the like. Suitable alkyls include, for example, methyl, ethyl, propyl, substituted alkyl (e.g., alkane with hydroxyl, hydroalkyl, amine, amide) or the like. Suitable branched alkyl can be, for example, isopropyl, isobutyl, substituted branched alkyl, or the like. Suitable cyclo-alkyls can include, for example, cyclohexane. cycloheptane, and other cyclic alkanes as well as substituted cyclic alkanes such as substituted cyclohexane or substituted cycloheptane. Suitable halogens include, for example, bromine, chlorine, fluorine, or the like. Suitable heteroatoms include, for example, sulfur, silicon, and fluoro-or bromo-substitutions. Suitable substituted alkyls, substituted branch alkyls and substituted cyclo-alkyls include, for example, alkyls, branched alkyls and cyclo-alkyls substituted with oxyeen, hydroxyl, nitrogen, amide, amine, halogen, a heteroatom or other groups. In exemplary embodiments, the synthetic retinoid is 9-ethyl-11-cis-retinal, 7-methyl-11-cis-retinal, 13-desmethyl-11-cis-retinal,11-cis-10-F-retinal, 11-cis-10-C1-retinal, 11-cis-10-methyl-retinal, 11-cis-10-ethyl-retinal, 9-cis-10-F-retinal, 9-cis-10-C1-retinal, 9-cis-10-methyl-retinal, 9-cis-10-ethyl-retinal, 11-cis-12-F-retinal, 11-cis-12-C1-retinal, 11-cis-12-methyl-retinal, 11-cis-

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10-ethyl-retinal, 9-cis-12-F-retinal, 9-cis-12-C1-retinal, 9-cis-12-methyl-retinal, 11-cis-14-F-retinal, 11-cis-14-methyl-retinal, 11-cis-14-ethyl-retinal, 9-cis-14-F-retinal, 9-cis-14-methyl-retinal, 9-cis-14-ethyl-retinal, or the like.
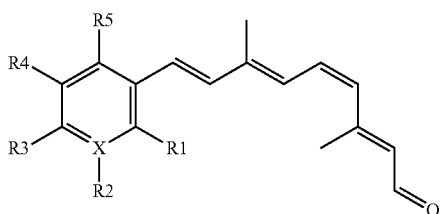
The synthetic retinoid further can be derivative of an 11-cis-retinal or 9-cis-retinal that has a modified ring structure. Suitable examples include, for example, derivatives containing ring modifications, aromatic analogs and heteroaromatic analogs of the following formulae IV, V and VI, respectively:


(IV)
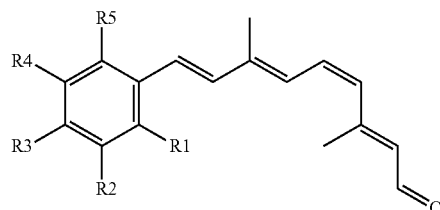
(V)

(VI)

Each of R1 to R5 or R6, as applicable, can be independently selected from hydrogen, alkyl, substituted alkyl, hydroxyl, hydroalkyl, amine, amide, halogen, a heteratom, or the like. Suitable alkyls include, for example, methyl, ethyl, propyl, isopropyl, butyl, isobutyl or the like. Suitable halogens include, for example, bromine, chlorine, fluorine, or the like. Suitable heteroatoms include, for example, sulfur, silicon, or nitrogen. In addition, X can be a heteroatoms, such as, for example, sulfur, silicon, or nitrogen.

The synthetic retinoid can further be a derivative of an 11-cis-retinal or 9-cis-retinal that has a modified polyene chain. Suitable derivatives include, for example, those with a translcis locked configuration, 6s-locked analogs, as well as modified allene, alkene, alkyne or alkylene groups in the polyene chain. In one example, the derivative is an 11-cis-locked analog of the following formula VII:
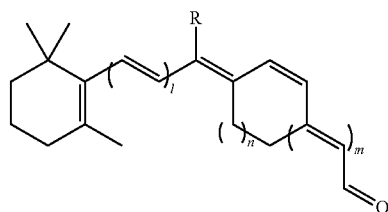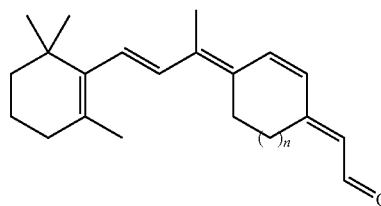

(VII)

R can be, for example, hydrogen, methyl or other lower alkane or branched alkane, or a heteroatom. n can be 0 to 4, m plus 1 equals 1, 2 or 3.

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In a specific embodiment, the synthetic retinoid is a 11-cis-locked analog of the following formula VIII:

(VIII)

n can be 1 to 4.

In certain exemplary embodiments, the synthetic retinoid is 9,11,13-tri-cis-7-ring retinal, 11,13-di-cis-7-ring retinal, 11-cis-7-ring retinal or 9,11-di-cis-7-ring retinal.

In another example, the synthetic retinoid is a 6s-locked analog of formula IX. R1 and R2 can be independently selected from hydrogen, methyl and other lower alkyl and substituted lower alkyl. R3 can be independently selected from an alkene group at either of the indicated positions.

(X)

(XI)

(IX)
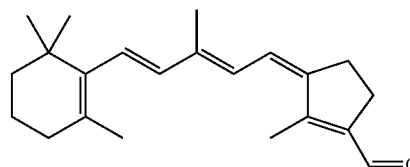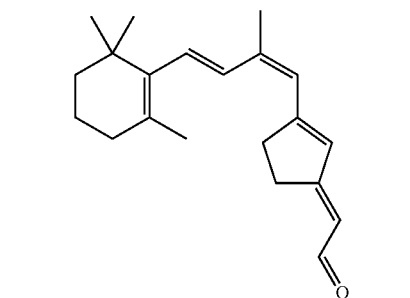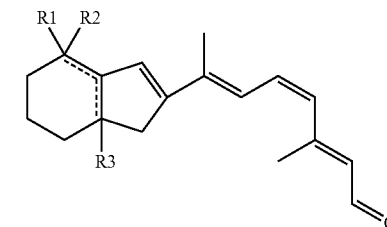
(XII)

In other embodiments, the synthetic retinoid can be a 9-cis-ring-fused derivative, such as, for example, those shown in formulae X-XII.

In yet another embodiment, the synthetic retinoid is of the following formula XIII.

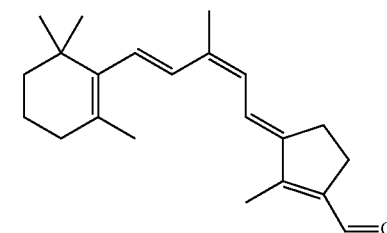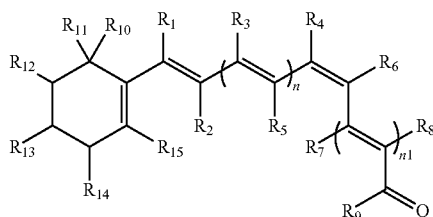Each of R1 to R15 can be independently selected from hydrogen, alkyl, branched alkyl, halogen, hydroxyl, hydroxyalkyl, amine, amide, a heteroatom, or the like. Suitable alkyls include, for example, methyl, ethyl, propyl, substituted alkyl (e.g., alkyl with hydroxyl, hydroxyalkyl, amine, amide), or the like. Suitable branched alkyl can be, for example, isopropyl, isobutyl, substituted branched alkyl, or the like. Suitable halogens include, for example, bromine, chlorine, fluorine, or the like. Suitable heteroatoms include, for example, sulfur, silicon, and fluoro- or bromo- substitutions. Suitable substituted alkyls and substituted branch alkyls include, for example, alkyls and branched alkyls substituted with oxygen, hydroxyl, nitrogen, amide, amine, halogen, heteroatom or other groups. Each of n and n_1 can be independently selected from 1, 2, or 3 alkyl, alkene or alkylene groups, with the proviso that the sum of the n and n_1 is at least 1. In addition, R11-R12 and/or R13-R14 can comprise an alkene group in the cyclic carbon ring. In certain embodiments, R5 and R7 together can form a cyclo-alkyl, such as a five, six, seven or eight member cyclo-alkyl or substituted cyclo-alkyl, such as, for example, those shown in formulae VII, VIII, X, XI and XII.

In additional embodiments, the synthetic retinoid also can be 9-cis-retinal. Alternatively, 11-cis-retinal can be used.

Methods of making synthetic retinoids are disclosed in, for example, the following references: *Anal. Biochem.* 272:232-42 (1999); *Angew. Chem.* 36:2089-93 (1997); *Biochemistry* 14:3933-41 (1975); *Biochemistry* 21:384-93 (1982); *Biochemistry* 28:2732-39 (1989); *Biochemistry* 33:408-16 (1994); *Biochemisity* 35:6257-62 (1996); *Bioorganic Chemistry* 27:372-82 (1999); *Biophys. Chem.* 56:31-39 (1995); *Biophys. J.* 56:1259-65 (1989); *Biophys. J.* 83:3460-69 (2002); *Chemistry* 7:4198-204 (2001); *Chemistry* (Europe) 5:1172-75 (1999); *FEBS* 158:1 (1983); *J. American Chem. Soc.* 104:3214-16 (1982); *J. Am. Chem. Soc.* 108:6077-78 (1986); *J. Am. Chem. Soc:.* 109:6163 (1987); *J. Am. Chem. Soc.* 112:7779-82 (1990); *J. Am. Chem. Soc.* 119:5758-59 (1997); *J. Am. Chem. Soc.* 121:5803-04 (1999): *J. American Chem. Soc.* 123:10024-29 (2001); *J. American Chem. Soc.* 124:7294-302 (2002); *J. Biol. Chem.* 276:26148-53 (2001); *J. Biol. Chem.* 277:42315-24 (2004); *J. Chem. Soc.—Perkin T.* 1:1773-77 (1997); *J. Chem. Soc.—Perkin T.* 1:2430-39 (2001); *J. Org. Chem.* 49:649-52 (1984); *J. Org. Chem.* 58:3533-37 (1993); *J. Physical Chemistry B* 102:2787-806 (1998); *Lipids* 8:558-65; *Phoiochem. Photobiol.* 13:259-83 (1986); *Pholochem. Photobiol.* 44:803-07 (1986); *Photochem. Photobiol.* 54:969-76 (1991); *Photochem. Pholobiol.* 60:64-68 (1994): *Photochem. Photobiol.* 65:1047-55 (1991): *Photochem. Photobiol.* 70:111-15 (2002); *Pholochem. Phombiol.* 76:606-615 (2002): *Proc. Natl Acad. Sci. USA* 88:9412-16 (1991): *Proc. Natl. Acad Sci. USA* 90:4072-76 (1993); *Proc. Natl Acad. Sci. USA* 94:13442-47 (1997); and

Proc. R. Soc. Lund. Series B, Biol. Sci. 233(1270): 55-76 1988) (the disclosures of which are incorporated by reference herein).

Opsin-binding synthetic retinoids can be identified by an expression system expressing a mutant opsin protein. Suitable expressing systems can include, for example, in vitro or in vivo systems. Suitable in vitro systems include for example, coupled transcription-translation systems. Suitable in vivo systems include, for example, animal models and cells expressing a mutant opsin protein. For example, cells of a vertebrate visual system can be adapted for culture in vitro, or recombinant cell lines expressing an opsin protein can he used.

Suitable non-human animal models include rat, mouse, primate systems. Such animal models can be prepared, for example, by promoting homologous recombination between a nucleic acid encoding an opsin in its chromosome and an exogenous nucleic acid encoding a mutant opsin. In one aspect, homologous recombination is carried out by transforming embryo-derived stem (ES) cells with a vector containing an opsin gene, such that homologous recombination occurs. followed by injecting the ES cells into a blastocyst, and implanting the blastocyst into a foster mother, followed by the birth of the chimeric animal (see, e.g., Capecchi, *Science* 244:1288-92 (1989)). The chimeric animal can be bred to produce additional transgenic animals.

Also, recombinant cell lines expressing a mutant opsin protein can be used. The cell lines are typically stable cell lines expressing the mutant opsin protein. Synthetic retinoid can be added to the cell culture media, and the cells are cultured for a suitable period of time to allow the production of opsin/rhodopsin. Opsin and/or rhodopsin can be isolated (e.g., by immunoaffinity). Isolated protein samples are examined to determine the amount of pigment formed, and absorbance maxima.

Recombinant cell lines expressing mutant opsin protein can be prepared by, for example, introducing an expression construct encoding a mutant opsin protein into a suitable cell line. Methods of introducing nucleic acids into vertebrate cells are disclosed in, for example. Sambrook et a, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press (Cold Spring Harbor, N.Y. 2001) (the disclosure of which is incorporated by reference herein). The expression construct typically includes a promoter operably linked to a nucleic acid encoding a mutant opsin protein, and optionally a termination signal(s). Nucleic acids encoding opsin can be obtained, for example, by using information from a database (e.g., a genomic or cDNA library), by polymerase chain reaction, or the like. For example opsin encoding nucleic acids can be obtained by hybridization. (See generally Sambrook el al. (supra).) In a specific embodiment, an opsin encoding nucleic acid can be obtained by hybridization under conditions of low, medium or high stringency.

In certain embodiments, opsin encoding nucleic acids can be obtained under conditions of high stringency hybridization. By way of example, and not limitation, procedures using conditions of high stringency are as follows: Prehybridization of filters containing DNA is carried out for 8 hours to overnight at 65° C. in buffer composed of 6× SSC, 50 mM Tris-HCl (pH 7.5), 1.mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.02% BSA, and 500 µg/ml denatured salmon sperm DNA. Filters are hybridized for 48 hours at 65° C. in prehybridization mixture containing 100 µg/ml denatured salmon sperm DNA and 5-20×10^6 cpm of ^{32}P-labeled probe. Washing of filters is done at 65° C. for 1 hour in a solution containing 2× SSC, 0.01% PVP, 0.01% Ficoll, and 0.01% BSA. This is followed by a wash in 0.1× SSC at 50° C. for 45 minutes before

autoradiography. Other conditions of high stringency which can he used are well known in the art. (See generally Sambrook et al. (supra).)

The expression construct can optionally include one or more origins of replication and/or selectable marker(s) (e.g., an antibiotic resistance gene). Suitable selectable markers include, for example, those conferring resistance to ampicillin, tetracycline, neomycin, G418, and the like. Suitable cells lines include, for example. HEK293 cells, T-REx™-293 cells, CHO cells and other cells or cell lines.

The UV-visible spectra of rhodopsin mutants (comprising mutant opsin and a synthetic retinoid) can be monitored to determine whether the synthetic retinoid has formed a Schiff base with the mutant opsin protein. For example, acid-denatured, purified protein can be analyzed to determine whether an absorbance maxima of approximately 440 nm is present, providing evidence that the synthetic retinoid forms a Schiff base with the mutant opsin protein. In additional embodiments. hydroxylamine treatment can be used to confirm the Schiff's base is sequestered from the external environment (infra).

Suitable opsin-binding synthetic retinoids also can be selected by molecular modeling of rhodopsin and opsin mutants. The coordinates for rhodopsin crystal structure are available from the Protein Data Bank (1HZX) (Teller et al., *Biochemistry* 40:7761-72 (2001)). The effects of amino acid substitutions on the structure of rhodopsin, and on the contacts between opsin and 11-cis-retinal, or a synthetic retinoid, can be determined by molecular modeling.

In an exemplary embodiment, the coordinates for the rhodopsin crystal structure from the Protein Data Bank (1HZX) (Teller el al., *Biochemistry* 40:7761-72 (2001)) are used to generate a computer model. As appropriate, amino acid substitutions can be introduced into the rhodopsin crystal structure. The addition of hydrogen atoms and optimization can be done, for example, using Insight II (InsightII release 2000, Aceelrys, Inc., San Diego, Calif.). Crystallographic water can be removed, and water molecules introduced based on the accessible space in the extracellular region. Typically, no minimization is performed before water is added. A water layer (e.g., 5 Å thick) can be used to coat the extracellular part of rhodopsin as well as residues in contact with polar phospholipids heads. All of the water molecules can be allowed to move freely, as is the extracellular half of rhodopsin, with retinal. If no water cap is put on the cytoplasmic part of rhodopsin, this part of the molecule can be frozen to prevent degradation of the model.

In certain embodiments, a water cap is put on the extracellular part of rhodopsin (together with that part buried in membrane in contact with polar heads of phospholipids). Water and the extracellular part of rhodopsin can be allowed to move and the movement modeled at any suitable frequency. For example, the movement of the modeled rhodopsin can be modeling at 100 ps simulations.

Opsin-binding synthetic retinoids are contacted with a mutant opsin protein under conditions suitable and for a period of time sufficient for the formation of a mutant opsin protein/synthetic retinoid complex. The stability of the mutant opsinisynthetic retinoid complex can he determined by methods described herein or as known to the skilled artisan. The mutant opsin in the mutant opsinisynthetic retinoid complex is stabilized when it exhibits increased stability (e.g., increased affinity for the synthetic retinoid as compared with 11-cis-retinal, is less sensitive to hydroxylamine, exhibits less accumulation in ageresornes, or the like), as compared with the mutant opsin protein associated with 11-cis-retinal.

In certain embodiments, the mutant opsin protein is Pro23His opsin (i.e., having a proline to histidine amino acid substitution in opsin protein at position 23). The opsin-binding synthetic retinoid can be contacted with the mutant opsin protein in vitro or in vivo. For example, the mutant opsin protein can he synthesized in an in vitro translation system (e.g., a wheat germ or reticulocyte lysate expression system) and the synthetic retinoid added to the expression system. In additional embodiments, the mutant opsin protein can be contacted with the mutant opsin protein ex vivo, and then the complex can be administered to a vertebrate eye.

As used herein, "prophylactic" and "prophylactically" refer to the administration of a synthetic retinoid to prevent deterioration or further deterioration of the vertebrate visual system, as compared with a comparable vertebrate visual system not receiving the synthetic retinoid. The term "restore" refers to a long-term (e.g., as measured in weeks or months) improvement in photoreceptor function in a vertebrate visual system, as compared with a comparable vertebrate visual system not receiving the synthetic retinoid. The term "stabilize" refers to minimization of additional degradation in a vertebrate visual system, as compared with a comparable vertebrate visual system not receiving the synthetic retinoid.

Opsin-binding synthetic retinoids can be administered to a vertebrate eye having mutant opsin protein. In certain aspects, the vertebrate eve is characterized as having an inherited retinal disease, such as, for example, Retinitis Pigmentosa, in which there is a defect in opsin gene or opsin protein. In certain embodiments, the mutant opsin protein has a P23H amino acid substitution. In other embodiments, the mutant opsin protein has an amino acid substitution or other mutation in the N-terminal plug of opsin. The synthetic retinoid binds to and stabilizes the opsin protein. Typically, the stabilized opsin protein can be incorporated into the membrane rather than forming an aggregate.

Synthetic retinoids can be administered to human or other non-human vertebrates. Synthetic retinoids can be delivered to the eye by any suitable means, including, for example, oral or local administration. Modes of local administration can include, for example, eye drops, intraocular injection or periocular injection. Periocular injection typically involves injection of the synthetic retinoid into the conjunctiva or to the tennon (the fibrous tissue overlying the eye). Intraocular injection typically involves injection of the synthetic retinoid into the vitreous. In certain embodiments, the administration is non-invasive, such as by eye drops or oral dosage form.

Synthetic retinoids can be formulated for administration using pharmaceutically acceptable vehicles as well as techniques routinely used in the art. A vehicle is selected according to the solubility of the synthetic retinoid. Suitable ophthalmological compositions include those that are administrable locally to the eye, such as by eye drops, injection or the like. In the case of eye drops, the formulation can also optionally include, for example, isotonizing agents such as sodium chloride, concentrated glycerin, and the like; buffering agents such as sodium phosphate, sodium acetate, and the like; surfactants such as polyoxyethylene sorbitan monooleate (also referred to as Polysorbate 80), polyoxyl stearate 40, polyoxyethylene hydrogenated castor oil, and the like: stabilization agents such as sodium citrate. sodium edentate, and the like; preservatives such as benzalkonium chloride, parabens, and the like: and other ingredients. Preservatives can be employed, for example, at a level of from about 0.001 to about 1.0% weight/volume. The pH of the formulation is usually within the range acceptable to ophthalmologic formulations, such as within the range of about pH 4 to 8.

For injection, the synthetic retinoid can be provided in an injection grade saline solution, in the form of an injectable liposome solution, or the like. Intraocular and periocular injections are known to those skilled in the art and are described in numerous publications including, for example, *Ophthalmic Surgery: Principles of Practice*. Ed., G. I. Spaeth. W. B. Sanders Co. Philadelphia. Pa. U.S.A., pages 85-87 (1990).

Suitable oral dosage forms include, for example, tablets, pills, sachets, or capsules of hard or soft eelatin, methylcellulose or of another suitable material easily dissolved in the digestive tract. Suitable nontoxic solid carriers can be used which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. (See, e.g., Remington "*Pharmaceutical Sciences*", 17 Ed. Germano (ed.). Mack Publishing Co. Easton, Pa. (1985)).

The doses of the synthetic retinoids can be suitably selected depending on the clinical status, condition and age of the subject, dosage form and the like. In the case of eye drops, a synthetic retinoid can be administered, for example, from about 0.01 mg, about 0.1 mg, or about 1 mg, to about 25 mg, to about 50 mg, to about 90 mg per single dose. Eye drops can be administered one or more times per day, as needed. In the case of injections, suitable doses can be, for example, about 0.0001 mg, about 0.001 mg, about 0.01 mg, or about 0.1 mg, to about 10 mg, to about 25 mg, to about 50 mg, or to about 90 mg of the synthetic retinoid, one to four times per week. In other embodiments, about 1.0 to about 30 mg of synthetic retinoid can be administered one to three times per week.

Oral doses can typically range from about 1.0 to about 1000 mg, one to four times, or more, per day. An exemplary dosing range for oral administration is from about 10 to about 250 mg one to three times per day.

The following examples are provided merely as illustrative of various aspects of the invention and shall not be construed to limit the invention in any way.

EXAMPLES

Example 1

The present study demonstrates that P23H-opsin can he induced to properly fold by providing cells with a seven-membered ring variant of 11-cis-retinal during opsin biosynthesis. The affinity and selectivity of mutant opsin for 11-cis-7-ring retinal can he explained based on the crystal structure of rhodopsin.

Methods and Materials

Synthesis of 11-cis-7-Ring Retinals: Synthesis of 11-cis-7-ring retinals was performed as described previously with some modifications (Fujimoto et at., *Chirality* 14:340-46 (2002); Akito el al., *J. Am. Chem.* 102:6370-72 (1980); Caldwell et al., *J. Org. Chem.* 58:3533-37 (1993). All of the reactions were performed in a dried nitrogen atmosphere unless otherwise specified. 2-Cycloheptenone was first converted into allyl acetate by N-bromosuccinimide bromination in CCl$_4$ followed by treatment with KOAc in hexamethylphosphoramide. Purified 4-acetoxy-2-cycloheptenone (46% from 2-cycloheptenone) was subjected to a Horner-Emmons reaction with diethyl (2-cyanoethyl)phosphonate, which gave an isomeric mixture of two translcis (E/Z) cyanoacetates in a 2:1 ratio. The mixture was hydrolyzed with K$_2$CO$_3$ in MeOH:H$_2$O (5:1), and then the hydroxy group of the resulting allylic alcohol was protected with tert-butyldimethylsilyl chloride in pyridine (80% from cycloheptenonyl

acetate). The resulting cyano compound. was reduced with diisobutylaluminium hydride in CH$_2$Cl$_2$ to an aldehyde and purified by flash chromatography on a silica gel (63%). β-Cy-clocitral was reduced with NaBH$_4$ to β-cycloaeraniol and then reacted with triphenylphosphine hydrobromide in MeOH over 3 days to afford β-cyclogeranyltriphenylphosphonium bromide after the removal of solvent and drying of the residue in vacuum. Wittig, reaction of the silylated aldehyde with an excess of phosphonium salt in the presence of potassium tert-butoxide and a catalytic amount of 18-crown-6 in methylene chloride at ambient temperature afforded protected cyclic alcohol in 75% yield. The tert-butyldimethylsilyl protecting group was removed by treatment with tetrabutylammonium fluoride in dry THF, and the resulting alcohol was oxidized with MnO$_2$ in CH$_2$Cl$_2$ to a mixture of two (E/Z) cyclic ketones (2:1 ratio) in 96% yield. This mixture was condensed with tricthvl phosphonoacetate under Horner-Emmons conditions. followed by lithium aluminum hydride reduction of the resulting isomeric mixture of ethyl 7-ring retinoates and oxidation of retinols with MnO$_2$ (86%) in CH$_2$Cl$_2$.

The following isomers were used: isomer 1 (FIG. 1B). 9,11,13-tri-cis-7-ring retinal: 1.04 (s. 6H. 2×CH$_3$-1), 1.45-1.50 (m, 2H, CH$_2$-2), 1.59-1.67 (m, 2H, CH$_2$-3), 1.74 (s, 3H, CH$_3$-5), 1.90 (m, 2H, J 6.74 Hz, CH$_2$-22), 1.97 (s, 3H, CH$_3$-9), 2.03 (t, 2H, CH$_2$-4), 2.46 (t, 2H, J 7.26 Hz CH$_2$-20), 2.51 (t, 2H, J 6.74 Hz CH$_2$-21), 5.78 (d, 1H, J 7.78 Hz, H-14), 6.20 (d, 1H, J 11.42 Hz, H-7), 6.54 (d, 1H, J 11.42 Hz, H-8), 6.95 (d, 1H, J 16.08 Hz, H-12), 7.08 (d, 1H, J 16.08 Hz, H-11), 10.11 (d, 1H, J 7.78 Hz, H-15): isomer 2 (FIG. 1B), **11,13-di-cis-7-ring** retinal: 1.04 (s, 6H-2×CH$_3$-1), 1.47-1.50 (m, 2H, CH$_2$-2), 1.60-1.67 (m, 2H, CH$_2$-3), 1.73 (s, 3H, CH$_3$-5), 1.86 (m, 2H, J 6.74 Hz, CH$_2$-22), 2.00 (s, 3H, CH$_3$-9), 2.04 (t, 2H, CH$_2$-4), 2.45 (t, 2H, J 7.27 Hz CH$_2$-20), 2.54 (t, 2H, J 6.74 Hz CH$_2$-21), 5.79 (d, 1H, J 7.78 Hz, H-14), 6.32 (d, 1H,16.08 Hz, H-7), 6.52 (d, 1H, J 16.08 Hz, H-8), 7.02 (m, 2H, H-11, H-12), 10.12 (d, 1H, J 7.78 Hz, H-15); isomer 3 (FIG. 1B), 11-cis-7-ring retinal: 1.04 (s, 6H, 2×CH$_3$-1), 1.44-1.52 (m, 2H, CH$_2$-2), 1.57-1.67 (m, 2H, CH$_2$-3), 1.74 (s, 3H, CH$_3$-5), 1.86 (m, 2H, J 6.74 Hz, CH$_2$-22), 2.00 (s, 3H, CH$_3$-9), 2.04 (t, 2H, CH$_2$-4), 2.58 (t, 2H, J 6.75 Hz CH$_2$-20), 2.87 (t, 2H, J 6.75 Hz CH$_2$-21), 5.93 (d, 1H, J 8.3 Hz, H-14), 6.22 (d, 1H, J 11.42 Hz, H-12), 6.32 (d, 1H, J 16.08 Hz, H-7), 6.52 (d, 1H, J 16.08 Hz, H-8), 6.91 (s, 1H, J 11.42 Hz, H-11), 10.03 (d, 1H, J 7.79 Hz, H-15); and isomer 4 (FIG. 1B), 9,11-di-cis-7-ring retinal: 1.04 (s, 6H, 2×CH$_2$-1), 1.44-1.52 (m, 2H, CH$_2$-2), 1.57-1.67 (m, CH$_2$-3), 1.74 (s, 3H, CH$_3$-5), 1.86 (m, 2H, J 6.74 Hz, CH$_2$-22), 2.00 (s, 3H, CH$_3$-9), 2.04 (t, 2H, CH$_2$-4), 2.58 (t, 2H, J 6.75 Hz CH$_2$-20), 2.87 (t, 2H, J 6.75 Hz CH$_2$-21), 5.93 (d, 1H, J 8.3 Hz. H-14), 6.17 (d, 1H, J 11.41 Hz, H-12), 6.21 (d, 1H, J 16.08 Hz, H-7), 6.53 (d, 1H, J 16:08 Hz, H-8), 6.98 (s, 1 H, J 11.41 Hz, H-11), 10.03 (d, 1H, J 7.78 Hz, H-15).

Cell Lines and Growth Conditions: For the construction and selection of tetracycline-inducible opsin cell lines, an Invitrouen T-REx™ system was used. Briefly, nucleic acids encoding wild-type opsin and the P23-H mutant were excised as EcoRI-NotI fragments from pMT4 and then cloned into the EcoRI-NotI site within the polylinker of pcDNA4. Using opsin-specific forward and reverse primers, the entire gene was sequenced for verification. These plasmids were then separately transfected by calcium-phosphate precipitation into T-REx™-293 cells that already stably expressed the tetracycline repressor; these cells were routinely grown under biasticidin selection. After transfection, zeocin was added to the culture medium, and survivine colonies of cells were isolated and subsequently expanded into larger 6-well plates. Each of these clones was exposed to tetracycline, and 48

hours after induction. the cells were harvested and solubilized with 1% DM. Separately, uninduced cells were also solubilized. The samples were run on SDS-PAGE and immunoblotted with the monoclonal antibody ID4. Cell lines were chosen on the basis of production of the least amount of opsin (i.e., nondetectable opsin levels on immunoblots) in the absence of tetracycline and moderate amounts of opsin in the presence of the drug. HEK293 cells were grown in Dulbecco's modified Eagle's medium containing high glucose (Invitrogen) at 37° C. in the presence of 5.0% CO_2. In all of the studies, the cells were harvested after 48 hours of induction with tetracycline (1 µg/ml).

Cell Culture and Regeneration: The cells were washed three times with PBS, harvested, and incubated with different analogs of 11-cis-retinal (50 µM) for 45 minutes at 4° C. The cells were then lysed with 1.0% n-dodecyl-maltoside (DM) (Anatrace) in the presence of protease inhibitors (complete protease inhibitor mixture tablets: Roche Molecular Biochemicals) and centrifuged at 36.000 rpm in a Beckman ultracentrifuge for 30 minutes at 4° C. The supernatant was incubated with ID4-coupled CNBr-activated Sepharose 4B (Pharmacia Corp.) beads overnight. The beads were then washed three times with PBS containing 0.1% DM. Bound pigments were eluted off the beads using 0.1 mM synthetic peptide (last 9 amino acid residues of the C terminus of rhodopsin) in 0.1% DM. In studies when retinoids (50 µM) were added during biosynthesis, a Me_2SO solution of retinoids (10 µl of 100 mM) was added directly to the cell culture medium after 2 and 24 hours of induction. The cells. were harvested at 48 hours and lysed with 1.0% DM. Pigment was purified by immunoaffinity chromatography. The UV-visible spectra of the eluted pigment samples were then recorded on a Perkin Elmer Lambda 800 UV-visible spectrophotometer in the range of 250-650 nm.

SDS Electrophoresis and Immunoblotting: The samples were loaded on 10% SDS-polyacrylamide gels and transferred to lmmobilon-NC membrane (Millipore) according to established protocols. The membranes were blocked with blocking buffer (Licor) for 1 hour and incubated at room temperature with ID4 antibody (1:1000) for 1 hour. The membranes were then washed with PBS containing 0.1% Tween 20 and then incubated for 1 hour at room temperature with IRDye800™-conjugated affinity purified goat anti-mouse IgG (Licor), diluted 1:5000. The membranes were then washed with PBS containing Tween 20 and scanned using an Odyssey Infrared imager (Licor).

Glycosylation Status: Purified 7-rhodopsin and 7-P23H-rhodopsin (rhodopsin and P23H-rhodopsin regenerated with 1-cis-7-ring retinal, respectively) were treated with N-glycanase according to the manufacturer's recommendations (Glyko). The samples were incubated at 37° C. for 16 hours and then loaded onto a 10% polyacrylamide gel. The immunoblots were developed as described above.

Photosensitivity Studies: Whole cells expressing wild-type and P23H-opsin treated with 11-cis-7-ring retinals were harvested and exposed for 20 minutes to light emitted from a Fiber-Lite. Ml 150. High Intensity Illuminator (Dolan-Jenner). The cells were then washed and then lysed with 1.0% DM as described above. In a separate study, immunoaffinity purified 7-rhodopsin and 7-P23H-rhodopsin samples were exposed to light from Fiber-Lite, and UV-visible spectra were recorded at different times.

Hydroxylantine Sensitivity: Whole cells expressing wild-type and P23H-opsin treated with 11-cis-7-ring retinals were harvested and treated with 500 mM neutral solution of hydroxylamine for 45 min. The cells were thoroughly washed with PBS to remove all traces of hydroxylamine. Rhodopsin

was then purified, and spectra were taken. In a separate study, purified 7-rhodopsin and 7-P23H-rhodopsin were treated with 20 mM neutral hydroxylamine, and UV-visible spectra were recorded at different times.

Thermostability of Pigments and Acid Hydrolysis: Purified 7-rhodopsin and 7-P23H-rhodopsin were incubated at 37° C., and the spectra were recorded at indicated times. In acid treatment studies, purified 7-rhodopsin and 7-P23H-rhodopsin were treated with 100 mM sulfuric acid, and spectra were recorded.

HPLC Analysis of Retinoids: A 450 µl aliquot of either purified 7-rhodopsin, purified 7-P23H-rhodopsin, or cell medium were treated with 50 µl of 10% SDS and 100 µl of 1 M NH_2OH (freshly prepared, pH 7.5). The mixture was kept at room temperature for 30 min, and then 400 µl of MeOH and 400 µl of hexane were added. The mixture was shaken on a vortex for 5 minutes and centrifuged at 14,000 rpm to separate layers. The hexane layer was collected. Another 400 µl of hexane was added to the aqueous layer, and extraction was repeated. Combined hexane layers were dried down and redissolved in 120 µl of hexane, and 100 µl fractions were analyzed by normal phase HPLC as previously described.

Immunocytochemistry: HEK293 cells expressing wild-type rhodopsin or P23H-opsin under a tetracycline-inducible promoter were cultured in Dulbecco's modified Eagle's medium (Invitrogen). The cells were attached to glass bottom microwell dishes (MatTek Corp.) with Cell-Tak (Becton Dickinson Labware). Expression of opsin or P23H-opsin was induced by the addition of 1 µg/ml tetracycline as recommended by the manufacturer's protocol (Invitrogen). The cells were treated with 50 µM 11-cis-7-ring retinal 2 hours after induction. The cells were harvested after 24 hours and fixed with 4% paraformaldehyde (Fisher) in PBS (136 mM NaCl, 11.4 mM sodium phosphate, pH 7.4) for 10 minutes and washed by PBS. To block nonspecific labeling, the cells were incubated in 1.5% normal goat serum (Vector Lab., Inc.) in PBST (136 mM NaCl, 11.4 mM sodium phosphate. 0.1% Triton X-100, pH 7.4) for 15 minutes at room temperature. The cells were incubated overnight at 4° C. in purified ID4 antibody diluted with POST. The sections were rinsed in POST and incubated with indocarbocyanine (Cy3)-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Lab. Inc.) and Hoechst 33342 dye (Molecular Probes). The cells were rinsed in PBST and mounted in 50 µl of 2% 1,4-diazabicyclo-[2.2.2]octane (Sigma) in 90% glycerol to retard photobleachine. For confocal imaeine, the cells were analyzed on a Zeiss LSM510 laser scanning microscope (Carl Zeiss, Inc.).

Protein Simulation and Modeling: Coordinates for rhodopsin were taken from the Protein Data Bank (1HZX) (Teller et al. *Biochemistry* 40:7761-72 (2001). The addition of hydrogen atoms and all of the optimizations were done in Insight II (InsightII release 2000, Accelrys, Inc., San Diego, Calif.). Crystallographic water was removed, and water molecules were introduced based on the accessible space in the extracellular region. No minimization was performed before water was added.

A water layer (5 Å thick) was used to coat the extracellular part of rhodopsin as well as residues in contact with polar phospholipids heads. All of the water molecules were allowed to move freely, as was the extracellular half of rhodopsin, which contains P23H and retinal. Because no water cap was put on the cytoplasmic part of rhodopsin, this part of the molecule was frozen to prevent degradation of the model.

Results

The chromophore of rhodopsin, 11-cis-retinal, plays a central role in the photoactivation process and is also important

for the stabilization of the receptor. For example, rhodopsin is stable for months in mild detergents, although opsin precipitates in a few hours. The chromophore may also induce a proper folding of rhodopsin mutants. The P23H amino acid substitution may destabilize the native chromophore-accepting conformation of opsin, leading to aberrant folding and subsequent aggregation. However, an analog of 11-cis-retinal may facilitate native-like folding of P23H-opsin and therefore play the role of pharmacological chaperone. Recently, wild-type opsin was regenerated with 11-cis-7-ring retinal (7-rhodopsin), containine a chromophore with a seven-membered ring to prevent isomerization around the C11=C12 double bond (FIG. 1B), is very stable in vivo and in vitro. In contrast, bleaching of the wild-type opsin regenerated with 11-cis-6-ring retinal (6-rhodopsin), which contains a more rigid chromophore, produces multiple isomers with modest changes in protein conformations. These results reveal that 11-cis-7-ring retinals, particularly isomer 3 (FIG. 1B), are easily accepted into the binding site of opsin and provide additional contact sites by virtue of the seven-membered ring and the 9-methyl group with the protein moiety. Because of its intrinsic flexibility, this analog could potentially influence/affect protein folding during biosynthesis, which is particularly important for those mutant proteins that fold less efficiently. The binding pocket of wild-type opsin adopts specific conformations that allow it to bind preferentially certain retinoids.

11-cis-7-Ring Retinal Chaperone Folding of P23H-opsin: Rhodopsin is regenerated with 11-cis-7-ring retinals in the harvested membranes (FIG. 2B), whereas only a small amount of pigment was formed when membranes containing P23H-opsin were treated with 11-cis-retinal. 11-cis-6-ring retinal, or 11-cis-7-ring retinal after the cells were harvested (FIG. 2B). If the mutant proteins are structurally unstable, it was proposed that the addition of retinal during the course of protein biosynthesis might induce a more native-like folding of the protein and its subsequent stabilization.

The first studies utilized 11-cis-locked versions of 11-cis-retinal in stable cell lines expressing wild-type and P23H-opsin. The UV-visible spectra of the immunoaffinity purified samples from 11-cis-6- or 11-cis-7-ring retinals provided to the cells during biosynthesis showed that both retinals recombined with wild-type opsin (FIG. 2C). Supporting this prediction. P23H-opsin formed significant amounts of pigment when 11-cis-7-ring retinal was added during biosynthesis (FIG. 2D). The max of the pigment was approximately 490±3 nm, unlike the wild-type protein (with a max of approximately 500±3 nm) (FIGS. 2, C and D). The UV-visible spectra with a max of approximately 440 nm of acid-denatured purified 7-P23H-rhodopsin (FIG. 2D, inset) provided evidence that the chromophore forms a Schiff base with rescued P23H-opsin. No significant pigment was seen when 11-cis-6-ring retinal, which is photoisomerable along C9- and C13-C=C double bonds, was added to P23H-opsin-expressing cells (FIG. 2D). Furthermore, 11-cis-9-demethyl-7-ring retinal was also ineffective in pigment formation with P23H-opsin. These studies illustrate unique specificity of the binding interaction between 11-cis-7-ring retinal and P23H-opsin.

Immunoblots showed that the rescued P23H-opsin had a high molecular weight and a Golgi-associated glycosylation pattern similar to the wild-type protein, suggesting that the protein is sufficiently folded to proceed along the secretory pathway. This is in striking contrast to P23H-opsin that was not treated with 11-cis-7-ring retinal during P23H-opsin biosynthesis. Purified 7-rhodopsin-and 7-P23H-rhodopsin were efficiently deglycosylated by N-glycanase, and the mutant protein spontaneously aggregated. forming high molecular weight aggregates).

In control studies, 9-cis-retinal and 11-cis-retinal were tested for proper folding of P23H-opsin. It should also be noted that 9-cis-retinal promoted transport of P23H-opsin to the cell surface, 11-cis-retinal was also observed to induce the in vivo folding and stabilization of P23H-opsin forming visual pigments (499 nm and 492 nm, for rhodopsin and P23H-rhodopsin, respectively) (FIGS. 2, C and D). Moreover, these pigments also acquired a mature state of glycosylation. 11-cis-retinoids are unstable in vitro and in vivo and rapidly undergo "reverse isomerization.". Therefore, locked analogs were used throughout the rest of this study.

Properties and Stability of 7-P23H-rhodopsin: When the mixture of the four 7-ring retinal isomers (FIG. 1B) was added to cells expressing opsin and P23H-opsin during biosynthesis, both wild-type opsin and P23H-opsin selectively bound only isomer 3. These observations further support the extraordinary specificity of wild-type and P23H-opsin for binding this 11-cis-retinal isomer. Addition of 11-cis-retinal to cell membranes containing either wild-type or P23H-opsin that were already treated with the 11-cis-7-ring retinal during their biosynthesis did not lead to its substitution after photobleaching, as measured spectrophotometrically and by retinoid analysis of the bound chromophores, 7-Rhodopsin. purified in the detergent, is stable to light (FIG. 3A), whereas purified 7-P23H-rhodopsin undergoes bleaching under the same conditions (FIG. 3B). In contrast, both rhodopsins are stable in the membranes of HEK293 cells (FIGS. 3, C and D). This result suggests that, in detergent, the chromophore in 7-P23H-rhodopsin is more flexible, thus allowing photoisomerization. The mechanism by which 7-P23H-rhodopsin undergoes bleaching in detergent most likely involves isomerization of isomer 3 to three other isomers and its subsequent hydrolysis. The membranes apparently stabilized 7-P23H-rhodopsin, thus preventing isomerization of its bound chromophore. In tissue-culture samples, small amounts of non-rhodopsin-bound retinals eluted between 30 and 40 minutes and remained in equilibrium with the vast majority of retinols, governed by the redox potential of the cells.

To further characterize the rescued protein, the affinity bound 7-rhodopsin and 7-P23H-rhodopsin were eluted under conditions that selectively released only the folded form of the protein (10 mM phosphate, pH 6.0). The spectra of the eluates (FIGS. 2, E and F) and the corresponding, immunoblots indicated that approximately 80% of 7-P23H-rhodopsin folded to form pigment. Additionally, immunoblots of these eluates demonstrated that both contained a mature glycosylated band and unglycosylated bands; however, only the material eluted at pH 6.0 had the Golgi-specific glycosylation pattern.

The structure of the rescued protein was also probed by determining its sensitivity to neutral hydroxylamine, 7-P23H-rhodopsin is resistant to hydroxylamine treatment when embedded in the lipid bilayer of HEK293 cells, suggesting that the rescued protein adopts a conformation that sequesters the Schiff base linkage within the protein and protects it from chemical modification. Unlike the purified wild-type protein, the mutant chromophore is accessible to hydroxylamine in detergent (0.1% DM). as evidenced by the formation of the retinaloximes (max=approximately 360 nm). These data imply that the structure of 7-P23H-rhodopsin is less tightly packed than that of the 7-rhodopsin. Finally, the temperature stability of the purified 7-rhodopsin and 7-P23H-rhodopsin was studied. There was no change in the amount of

chromophore after incubation of 7-rhodopsin at either 4° C. or 37° C. However, for the rescued 7-P23H-rhodopsin, there is time-dependent thermal bleaching that is accelerated by increased temperature, with a half-life of approximately 4 minutes at 37° C. and 10 days at 4° C. in the detergent solution.

Localization of the Rescued 7-Locked-P23H-Rhodopsin: Immunofluorescence microscopy demonstrated that P23H-opsin is retained intracellularly, predominantly in a peri-nuclear distribution with punctuated fluorescence consistent with aegresomes. In contrast, the rescued 7-P23H-rhodopsin is predominantly found in a diffuse pattern with significantly greater staining at the cell surface similar to the 7-rhodopsin and wild-type opsin, which are found predominantly at the plasma membrane. In summary, this result indicates that the rescued P23H-rhodopsin was not only correctly glycosylated and formed a pigment but was also correctly routed to the cell membranes.

Discussion

Protein Conformational Disorders: Dominantly inherited diseases can result from (a) haploinsufficiency of a gene, e.g., as for the melanocortin-4 receptor; (b) constitutive activity (gain of function) in some opsin mutants or lack of activity because of somatic mutation; and (c) loss of function because of mutant protein misfolding and aggregation. The diseases caused by misfolded proteins are known as protein conformational disorders and include Alzheimer's disease, Huntington's disease, Parkinson's disease, diabetes insipidus, cystic fibrosis, prion disease, emphysema, dominant cataracts, and oculopharyngeal muscular dystrophy. Gene delivery is one method of correcting haploinsufficiency. Constitutive activity has been successfully blocked by reverse agonist-like compounds, and conformation and function can be rescued by small chemical agents. The medically most prevalent forms of dominant disorders are the protein conformational disorders. Although they are the most challenging to treat, some successes have been reported. Examples include the rescue of misfolded cystic fibrosis transmembrane regulator (CFTR) variant F508 and the Z-variant of 1-antitrypsin. In the instance of CFTR. the osmolytes glycerol and trimethylamino-oxide have been used. The protein acquires mature glycosylation and is transported to the cell surface where it pumps Cl ions. The chemical chaperone 4-phenyl-butyric acid mediated a marked increase in secretion of α_1-antitrypsin. Recent studies, however, suggest that the rescued protein is less stable than the wild-type. Other approaches to inducing the folding of proteins have been developed. For example, mini-chaperones that prevent β-sheet formation prevent toxic aggregation of amyloid-β found in Alzheimer's disease and the PrP protein associated with prion disease.

P23H Rescue: This study demonstrates that P23H-opsin can be induced to properly fold and be stabilized with a seven-membered locked ring version of 11-cis-retinal, the inverse agonist of opsin. Furthermore, this model system provides a quantitative means to study the folding of this membrane protein. The rescued 7-P23H-rhodopsin contains a Schiff base linkage with Lys296, producing pigment with a max of approximately 490 nm (FIG. 2D) that can be used as a quantitative measure of the amount or correctly folded protein. Using chromatographic conditions that selectively elute the folded form of the protein, 11-cis-7-ring retinal leads to the rescue of approximately 80% of the P23H-opsin. This study also demonstrated that the rescued protein is not only folded but also proceeds along the secretory pathway. Like the wild-type protein, the rescued protein acquires the heterogeneous glycosylation associated with oligosaccharide pro-

cessing within the Golgi apparatus. Clearly, the protein is of a sufficiently native-like conformation and is not sequestered by the cellular quality control apparatus, at least that which exists at the level of endoplasmic reticulum.

Furthermore, the rescued protein is structurally different from the wild-type protein. The detergent-purified protein is light-sensitive, displays thermal instability, and is sensitive to neutral hydroxylamine. The bleaching of detergent-solubilized 7-P23H-rhodopsin occurs because of isomerization of double bonds along the polyene chain of the chromophore producing a mixture of isomers. However, in the membranes of the HEK cells, 7-P23H-rhodopsin is stable, and therefore. it is expected this pigment will also be stable in vivo.

Chemical and Structural Considerations of the Rescue: The mutant P23H protein is less stable because it is misfolded and is thus retained intracellularly. The rescue effect with 11-cis-7-ring retinal leads to increased amounts of the correctly folded mutant and is extraordinarily specific as shown by the lack of activity observed with several structural homologs (11-cis-9-demethyl-ring retinal and 11-cis-6-ring retinal). However, unlike the wild-type protein, purified 7-P23H-rhodopsin appears to be in a less compact conformation, as evidenced by its sensitivity to neutral hydroxylamine in detergent. It was also observed that the rescued protein spontaneously releases the locked retinoid over time. Previous studies have demonstrated that 11-cis-7-ring retinal is inherently more flexible than 11-cis-6-ring retinal. The rigidity of 11-cis-6-ring retinal may not allow the P23H-opsin binding pocket to accept this chromophore. Alternatively, P23H-opsin may bind 11-cis-6-ring retinal but that the resulting protein is less stable and maybe more susceptible to water-assisted hydrolysis of the Schiff base linkage, as it is in the case of 9-cis-retinal.

These molecular modeling studies provide an explanation for the defect caused by P23H mutation. Two alterations appear to be present in P23H mutant: (a) The mutation disrupts ionic interactions of Pro23, Gln182, and Glu184 on the tip of the plug between helices 4 and 5. Mutation of Pro to His leads to formation of conjugated hydrogen bonding that exposes the chromophore-binding site. This interaction stiffens the plug and possibly opens the binding site of opsin, allowing water to hydrolyze the Schiff base between the retinal chromophore and γ-amino group of Lys296. This prediction is consistent with a hipsochromic shift in the absorption maximum of the chromophore and accessibility to hydrolysis in the presence or absence of hydroxylamine, (b) There is a hydrophilic channel in the interior of the protein that could accommodate water molecules (FIG. 1E). In the model, rhodopsin was soaked with water on the external side, only including the part of the membranes that is charged and without constraints imposed on water molecules. During simulation, a route was established where water molecules drifted from one side to another (cytoplasmic). This route was along helix II and partly along helices I and III. The key residue facilitating this transport was Thr93 on helix II (close to Glu113). Collectively, these two effects may alter the electronic/dipole environment around the retinal, explaining the nearly 10-nm blue shift.

In summary, this study has shown using a cell culture model system that the retinitis pigmentosa mutant P23H-opsin can be induced to properly fold by providing cells with a seven-membered ring variant of 11-cis-retinal during opsin biosynthesis. Furthermore, the remarkable affinity and selectivity of mutant opsin for 11-cis-7-ring retinal on the basis of the crystal structure of rhodopsin.

The previous examples are provided to illustrate but not to limit the scope of the claimed inventions. Other variants of the

inventions will be readily apparent to those of ordinary skill in the art and encompassed by the appended claims. All publications, patents, patent applications and other references cited herein are hereby incorporated by reference.

What is claimed is:

1. A method of treating retinitis pigmentosa, comprising: administering 9-cis-retinal to a human subject with retinitis pigmentosa due to expression of a mutant opsin protein with a substitution of proline 23 by histidine, wherein the 9-cis-retinal is administered in an amount effective to bind the mutant opsin protein to ameliorate loss of photoreceptor function.

2. The method of claim 1, wherein administering comprises administering an ophthalmologic composition comprising the 9-cis-retinal.

3. The method of claim 1, wherein administering comprises administering the 9-cis-retinal in a pharmaceutically acceptable vehicle.

4. The method of claim 1, wherein administering comprises administering the 9-cis-retinal orally.

5. The method of claim 4, wherein administering comprises administering a dose of 9-cis-retinal of between about 1-1000 mg, one to four times per day.

6. The method of claim 1, wherein administering comprises administering the 9-cis-retinal by injection.

7. The method of claim 1, wherein administering comprises administering the 9-cis-retinal locally.

8. The method of claim 7, wherein administering comprises administering the 9-cis-retinal locally in the form of an eye drop.

9. The method of claim 7, wherein administering comprises administering the 9-cis-retinal more than once per day.

10. The method of claim 7, wherein administering comprises administering a dose of 9-cis-retinal of between about 0.01-90 mg per single dose.

11. The method of claim 7, wherein administering comprises administering the 9-cis-retinal locally by intraocular injection or by periocular injection.

12. The method of claim 1, wherein administering comprises administering an amount of the 9-cis-retinal effective to induce folding of the mutant opsin protein to form visual pigment.

13. A method of treating a human subject suffering from retinitis pigmentosa due to expression of a mutant opsin protein with a substitution of proline 23 by histidine, comprising:

treating, to improve photoreceptor function in the human subject, by contacting the mutant opsin protein, in an eye of the subject, with a synthetic retinoid to form an opsin/synthetic retinoid complex, and wherein the synthetic retinoid is 9-cis-retinal.

14. The method of claim 13, wherein said contacting is achieved via delivery of an ophthalmologic composition.

15. The method of claim 14, wherein the ophthalmologic composition is locally delivered.

16. The method of claim 15, wherein the composition is delivered locally in the form of an eye drop, an intraocular injection or a periocular injection.

17. The method of claim 15, wherein the composition is delivered locally more than once per day.

18. The method of claim 13, wherein contacting is achieved via delivery of an oral composition.

19. The method of claim 13, wherein contacting is achieved via injection of a solution.

20. The method of claim 13, wherein said contacting is effective to induce folding of the mutant opsin protein to form visual pigment.

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